Research article

Alteration in expression of the rat mitochondrial ATPase 6 gene during *Pneumocystis carinii* infection

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Abstract

Background: *Pneumocystis carinii* causes pneumonia in immunocompromised patients with a high morbidity and mortality rate, but the interaction between this organism and the host cell is not well understood. The purpose of this research was to study the response of host cells to *P. carinii* infection on a molecular level.

Results: The technique of mRNA differential display was used to detect genes whose expression may be affected by *P. carinii* infection. The nucleotide sequence of one differentially displayed DNA fragment was found to be identical to that of the rat mitochondrial ATPase 6 gene, which is a subunit of the F_0F_1-ATP synthase complex. A four-fold increase in expression of this gene was verified by Northern blot analysis of total RNA extracted from *P. carinii*-infected rat lung versus that from mock-infected rat lung. Localization of the cells containing ATPase 6 mRNA was accomplished by *in situ* hybridization. In sections of non-infected rat lung, these cells were found lining the distal parts of the respiratory tree and in apical areas of the alveoli. Histological location of these cells suggested that they were Clara cells and type II pneumocytes. This hypothesis was confirmed by co-localizing the mRNAs for ATPase 6 and surfactant protein B (SP-B) to the same cells by two-color fluorescent *in situ* hybridization.

Conclusions: The ATPase 6 gene is over expressed during *P. carinii* infection, and type II pneumocytes and Clara cells are the cell types responsible for this over-expression.

Background

Pneumocystis carinii causes pneumonia in immunocompromised patients with a high morbidity and mortality rate. However, the interaction between this organism and the host cell is not well understood. The target cell for *P. carinii* is believed to be the type I pneumocyte. After contact with the type I pneumocyte, *P. carinii* trophozoites anchor themselves to the host cell. It was found that the major surface glycoprotein of *P. carinii* up-regulates the expression of integrins on the surface of cul-

tured lung cells [1] to facilitate this attachment. *P. carinii* attachment to type I pneumocytes may also be mediated by laminin, vitronectin [2,3], or mannose [4].

Alveolar macrophages interact with *P. carinii* in the lung as the first line of defense against infection. The major surface glycoprotein of *P. carinii* has been shown to be a chemotactic factor for macrophages and monocytes *in vitro* [5]. The interaction of alveolar macrophages with *P. carinii* is mediated by fibronectin [6]. *P. carinii* organisms are phagocytized when they bind to mannose receptors on the surface of macrophages [7]. Alveolar macrophages have been shown to release TNF-α, prostaglandin E_2, and leukotriene B_4 upon interaction with *P. carinii* [8]. These compounds are potent modulators of pulmonary inflammation and lung injury [9]. These are early and important events in the acute response to infection and clearance of *P. carinii* organisms from the lung [10].

Although it is not certain whether *P. carinii* organisms attach to type II pneumocytes, type II pneumocytes do respond to *P. carinii* infections. Type II pneumocytes maintain the structural integrity of alveoli for gas exchange. They produce alveolar surfactant and replicate and differentiate into type I pneumocytes after lung injury [11]. Type II pneumocytes have been shown to increase the production of surfactant protein-A (SP-A) in patients with *P. carinii* pneumonia (PcP), and the increase of SP-A correlates with the organism load in the lung [12]. The secretion of phosphatidylcholine from type II cells has been found to be inhibited upon *P. carinii* infection [13], leading to a deficiency of phosphatidylglycerol and the loss of surfactant function in patients with PcP [14].

The purpose of this study was to detect alterations in host cell gene expression that occur in response to *P. carinii* infection. We have compared gene expression patterns in *P. carinii*-infected and mock-infected cells using mRNA differential display and found that the mitochondrial ATPase 6 gene is over-expressed in response to *P. carinii* infection in rats. We also found that type II pneumocytes and Clara cells are responsible for over-expression of the ATPase 6 gene in *P. carinii*-infected rat lung.

Results

In order to determine whether *P. carinii* infection causes increases in gene expression in host cells, experiments using mRNA differential display were performed. Human embryonic lung (HEL) cells were used as host cells and were inoculated with lung homogenate of dexamethasone-immunosuppressed and *P. carinii*-infected rats. A separate set of HEL cell cultures was inoculated with lung homogenate of dexamethasone-immunosuppressed, non-infected rats to serve as non-infected (mock-infected) control. Several PCR product bands were seen in the *P. carinii*-infected but not in the mock-infected lanes of the mRNA differential display gel. Three of these bands (Fig. 1), designated A4II, A6I, and A8I, were selected for further study. To identify the genes represented by the differentially displayed PCR products, the DNA was recovered from the gel, re-amplified, cloned, and sequenced. The Basic Local Alignment Search Tool (BLAST) was used to identify the sequences thus obtained. The A4II product was determined to be a 234-bp DNA fragment with sequence approximately 60% homologous to nucleotides 134 - 234 of the yeast mitochondrial ori 1 sequence (gb|K02488|YSCMTORIA). This product was not further investigated since it is not a human or rat gene. The A6I product was found to be a 332-bp fragment with sequence identical to nucleotides 177 - 508 of the rat ribosomal protein S19 gene (emb|X51707|RRRPS19). The 287-bp A8I product was 100 % identical to nucleotides 3421 - 3707 of the rat mitochondrial ATPase 6 gene (gb|J01435|RATMTCYOS). Since we sought to detect genes that were up-regulated in the human host cell line, HEL 299, in response to *P. carinii* infection, it was surprising that none of the three selected PCR product bands were of human origin.

To verify the expression pattern seen in the mRNA differential display, the cloned A6I and A8I fragments were used to probe Northern blots of RNAs derived from non-infected and *P. carinii*-infected rat lungs. The A6I probe reacted with a 0.8-kb RNA band, but the intensities of the RNA bands of samples from mock- and *P. carinii*-infected rat lungs were approximately the same. Therefore, the A6I product band was not further studied. The A8I probe reacted with an RNA band of approximately 0.7 kb (Fig. 2), which is the size of the rat ATPase 6 mRNA [15]. Both *P. carinii*-infected and mock-infected RNA samples showed the 0.7-kb band. The intensity of the band of the RNA sample from *P. carinii*-infected rat lung is approximately 4 times stronger than that of the sample from non-infected rat lungs based on densitometric analysis of the autoradiogram. The band intensities of the same blot probed with a fragment of the 18S rRNA gene were used as an internal control for variations in the amount of RNA loaded on the blot (Fig. 2). These results indicate that non-infected rat lungs have a basal level of ATPase 6 gene expression and that this expression is increased in *P. carinii*-infected lungs.

To verify over-expression of the ATPase 6 gene in rat lung tissue, *in situ* hybridization with digoxigenin-labeled riboprobes was performed. Slides, each of which contained lung tissue sections of both dexamethasone-immunosuppressed non-infected (NRL) and *P. carinii*-infected (PcIRL) rats, were probed with sense and anti-

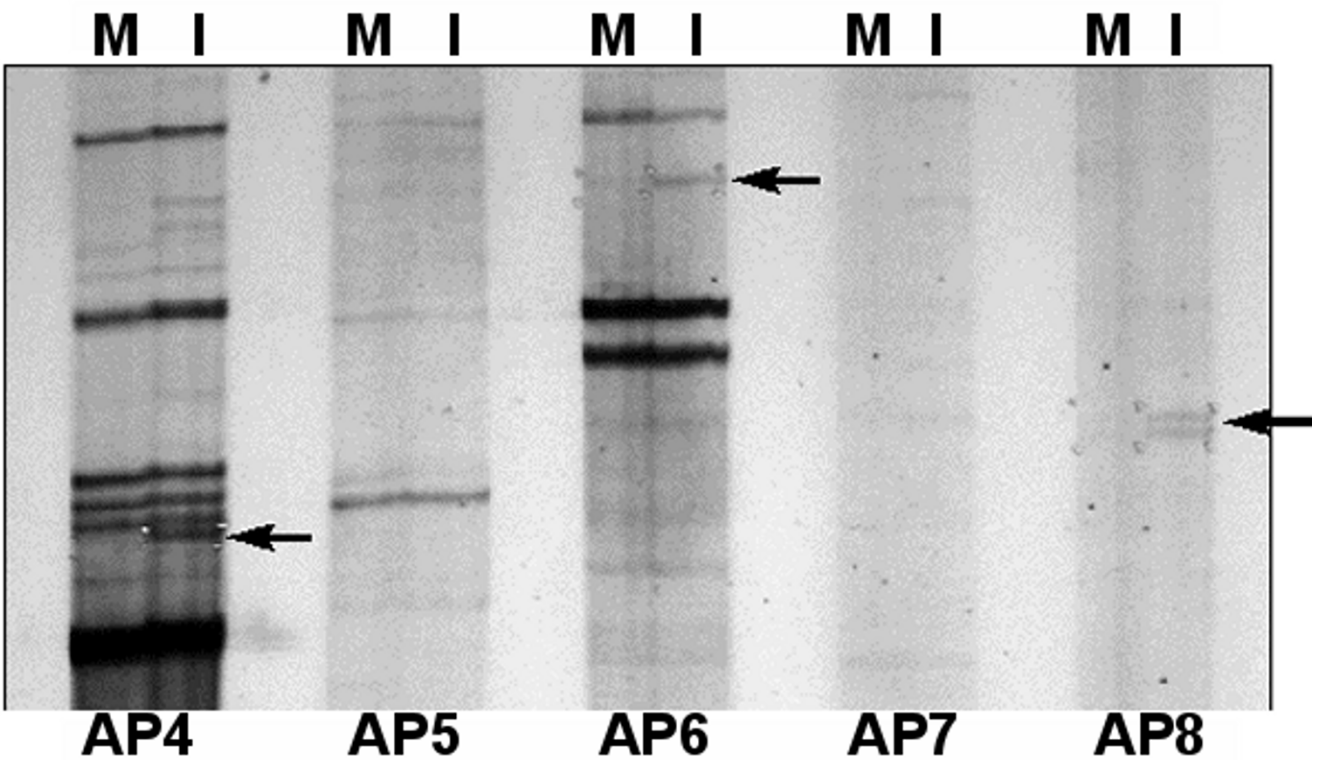

Figure 1
Products of mRNA Differential Display. Samples from mock-infected (M) and *P. carinii*-infected (I) cells were run in adjacent lanes on a 6% denaturing polyacrylamide gel. These PCR reactions contained the "A" anchored primer (H-T_{11}A) and one of the arbitrary primers (AP4, AP6, or AP8). The arrows indicate differentially displayed products A4II, A6I, and A8I.

sense riboprobes of the ATPase 6 gene. The sections reacted with the sense probe served as a control for non-specific background signal. The sections reacted with the anti-sense probe were expected to show a dark blue precipitate in cells as an indication of ATPase 6 gene expression. A dark blue precipitate was not seen in NRL and PcIRL sections reacted with the sense riboprobe (Fig. 3, ATPase 6 probe, panels NRL, S and PcIRL, S) but was seen in 2 - 3 cells per alveolus in the NRL sections reacted with the anti-sense riboprobe (Fig. 3, ATPase 6 probe, NRL, AS). Large alveolar macrophages with blue precipitates were also seen in some of the alveolar spaces. In addition, some of the epithelial cells of the very distal parts of the respiratory tree also showed blue precipitate (Data not shown). In sections of PcIRL reacted with the anti-sense riboprobe, hybridization signal was seen in many more cells per alveolus when compared to those of NRL. Some alveoli were packed with foamy exudate indicative of a heavy *P. carinii* infection. Most of the cells in the septal areas around these alveoli reacted with the probe (Fig. 3, ATPase 6 probe, PcIRL, AS), but there was no reaction with the probe in consolidated alveolar spaces, indicating that the probe did not react with *P. carinii* organisms.

Cells in the apical areas of the alveoli are usually type II pneumocytes, and the non-ciliated cuboidal epithelial cells in the very distal parts of the respiratory tree are usually Clara cells [16]. The observation that cells reacted with the ATPase 6 gene probe are located in these two areas suggests that type II pneumocytes and Clara cells are the cell types that over-expressed the ATPase 6 gene. To confirm this possibility, *in situ* hybridization with a probe specific for type II pneumocytes and Clara cells was performed. This probe was a 202-bp fragment of a portion of the rat surfactant protein-B (SP-B) gene (emb|X14778|RNSPB) that is expressed only in type II pneumocytes and Clara cells [17].

As seen in Fig. 3 (SPB probe, S), tissue sections (NRL or PcIRL) reacted with the SP-B sense probe showed no hybridization signal. Similarly to those probed with the ATPase 6 gene, cells in the apical area of alveoli (2-3 per alveolus) and epithelial cells lining the distal airways of NRL sections showed blue precipitate when reacted with the anti-sense SP-B probe (Fig. 3, SPB probe, NRL, AS). In PcIRL sections reacted with the anti-sense probe, more cells than in the NRL sections in apical areas of al-

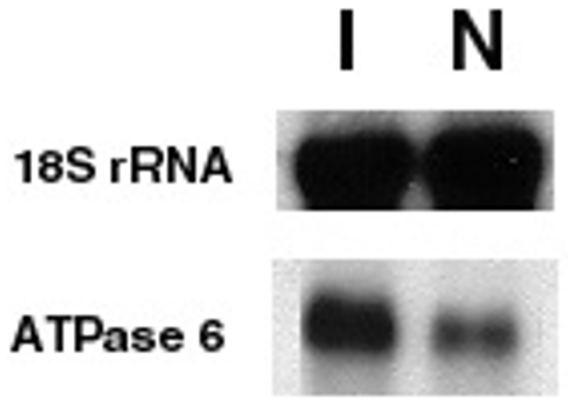

Figure 2
Northern Hybridizations Using the Cloned ATPase 6 Gene Fragment as Probe. Eight μg each of total RNAs derived from non-infected rat lung (N) and *P. carinii*-infected rat lung (I) were electrophoresed on an agarose gel containing formaldehyde, transferred to Nytran Plus membrane, and probed with a fragment of the 18S rRNA gene (control probe) and the mitochondrial ATPase 6 gene fragment.

veoli had blue precipitate, indicating SP-B expression (Fig. 3, SPB probe, PcIRL, AS).

To confirm that ATPase 6-expressing cells are type II pneumocytes and Clara cells, double-probe fluorescent *in situ* hybridization was performed. Sections of NRL and PcIRL tissues were hybridized simultaneously with fluorescein-labeled SP-B and digoxigenin-labeled ATPase 6 riboprobes. Hybridized fluorescein-labeled SP-B or digoxigenin-labeled ATPase 6 riboprobes were visualized as green or red fluorescence, respectively, using the appropriate filter on a fluorescent microscope. Cell nuclei were stained with DAPI and emitted a blue fluorescence under the appropriate filter. Sections of NRL and PcIRL hybridized with the sense probes for both ATPase 6 and SP-B showed little red or green fluorescence (Fig. 4, G and H). These sections exhibited predominately host cell nuclei that appeared as large blue elliptical rings or spherical masses, whereas *P. carinii* nuclei appeared as small blue, punctate clusters in the alveolar spaces (Fig. 4H). Composite images of NRL (Fig. 4A) and PcIRL (Fig. 4B) sections hybridized with the anti-sense riboprobe for both ATPase 6 and SP-B exhibited large blue nuclei surrounded by yellow fluorescence in the cytoplasm. This yellow fluorescence was due to complementation between red and green signals derived from anti-sense ATPase 6 (red signal) and SP-B (green signal) probes that hybridized with RNAs in the same cells. Panels 4, C and D; and E and F are images of 4A and 4B, respectively, split into individual green (SP-B) and red (ATPase 6) channels. With very rare exceptions, cells that expressed the ATPase 6 gene also expressed the SP-B gene. In addition, no hybridization signal (red or green) was seen in areas that had clusters of *P. carinii* organisms. These results strongly indicate that type II pneumocytes and Clara cells are the sources of ATPase 6 over-expression during *P. carinii* infection.

Discussion

The technique of mRNA differential display has been used to detect differences in gene expression between different types of cells [18,19], during cell transformation [20,21], and in various stages of disease development [22]. In this study, we used it to detect genes that are over-expressed in *P. carinii*-infected cells. Homogenates of lungs from non-infected and *P. carinii*-infected rats were used to inoculate a human cell line, the HEL cell line, to determine whether *P. carinii* would up-regulate or induce gene expression in infected HEL cells. Unexpectedly, we detected and identified the mitochondrial ATPase 6 gene, which appears to be over-expressed in response to *P. carinii* infection as demonstrated by Northern blot analysis (Fig. 2) and *in situ* hybridization (Figs. 3 and 4) in rat lungs. The possibility that the observed increase in hybridization signal to the rat ATPase 6 probe was due to crossover contamination by yeast- or *P.carinii*-derived ATPase 6 mRNA is remote because the mitochondrial ATPase 6 gene of yeast shares only 16 % sequence identity with that of rat. Furthermore, there was no indication of ATPase 6 signal originating from yeast or *P. carinii* organisms in the *in situ* experiments that were performed (Figs. 3 and 4).

ATPase 6 is a component of the F_0F_1 ATP synthase complex, which converts the energy stored in the form of the proton motive force to chemical energy in the form of ATP. This complex is composed of 2 major domains, F_1 and F_0. F_1 is the catalytic portion of the enzyme. It lies on the matrix side of the inner mitochondrial membrane and is composed of 6 subunits of $\alpha_3\beta_3\gamma\delta\varepsilon$ [23, 24] and an ATPase inhibitor protein [25]. F_0 is the integral membrane domain of the complex and functions as a proton channel in ATP synthesis. F_0 has 2 domains: the stalk-like domain and the integral membrane domain. The stalk-like domain of F_0 is composed of two proteins, OSCP (oligomycin sensitivity-conferring protein) [26] and F_6 [27]. OSCP is found as a dimer in each F_0 sector. It interacts with the F_1 sector and is required for H^+ translocation [28]. The integral membrane domain is composed of 3 main proteins: subunits 6, A6L, and 9. Subunit 6 is the product of the ATPase 6 gene. Little is known about the structure and function of the subunit 6 protein except that it is an integral membrane protein that interacts with the OSCP subunit and may be involved in energy coupling to the F_1 sector. The A6L sub-

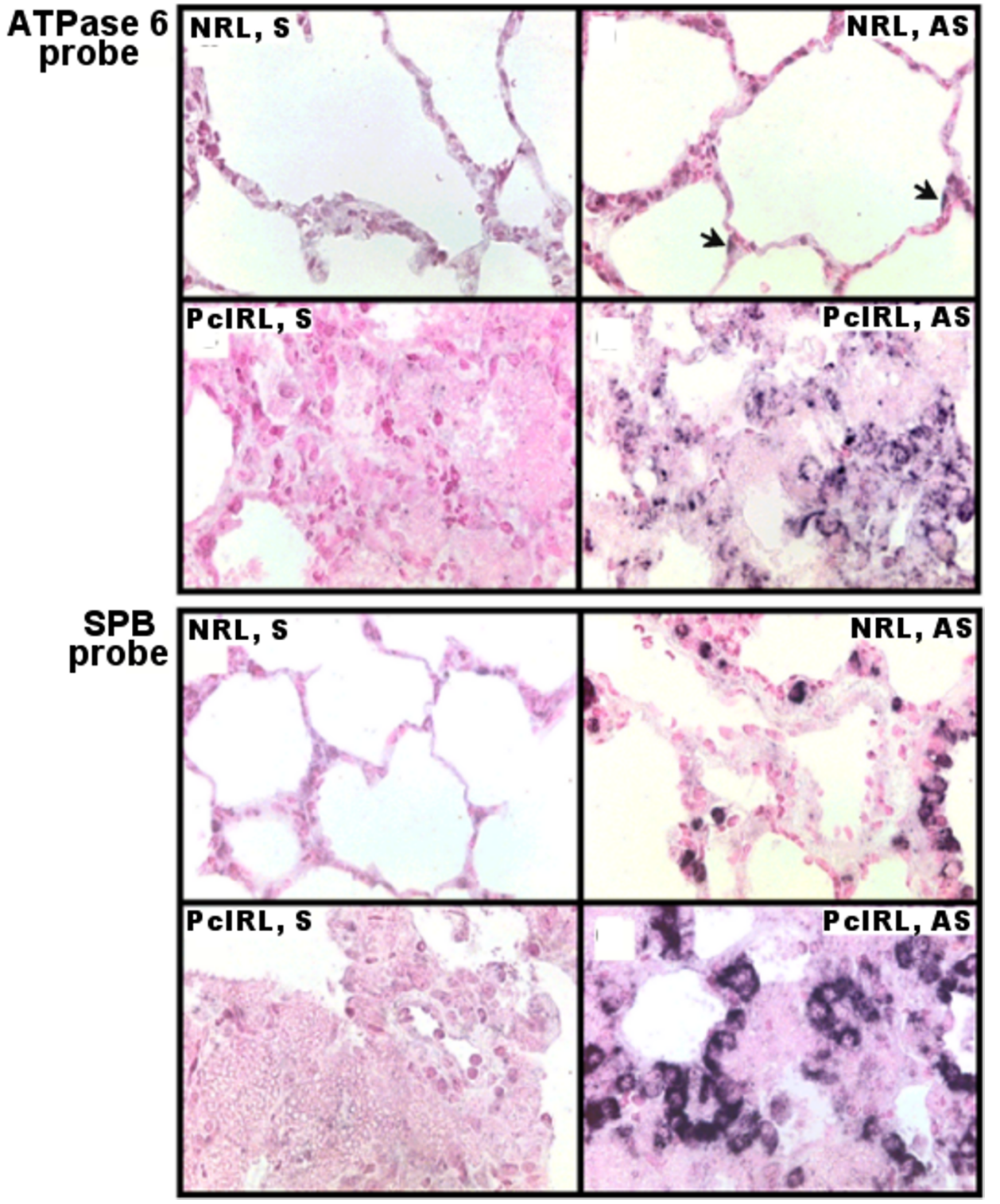

Figure 3
***in situ* Hybridizations Using Digoxigenin-labeled Riboprobes for ATPase 6 (A) and SP-B (B) with Colorimetric Detection.** Three micrometer-thick sections of dexamethasone-suppressed non-infected rat lung (NRL) and Pc-infected rat lung (PcIRL) were hybridized with ATPase 6 or SP-B probes. Left-side panels are representative sections probed with sense probes (S). Right-side panels show representative sections probed with anti-sense probes (AS). Arrows indicate cells that show the blue precipitate (indicating ATPase 6 expression) in the section of NRL probed with AS probe.

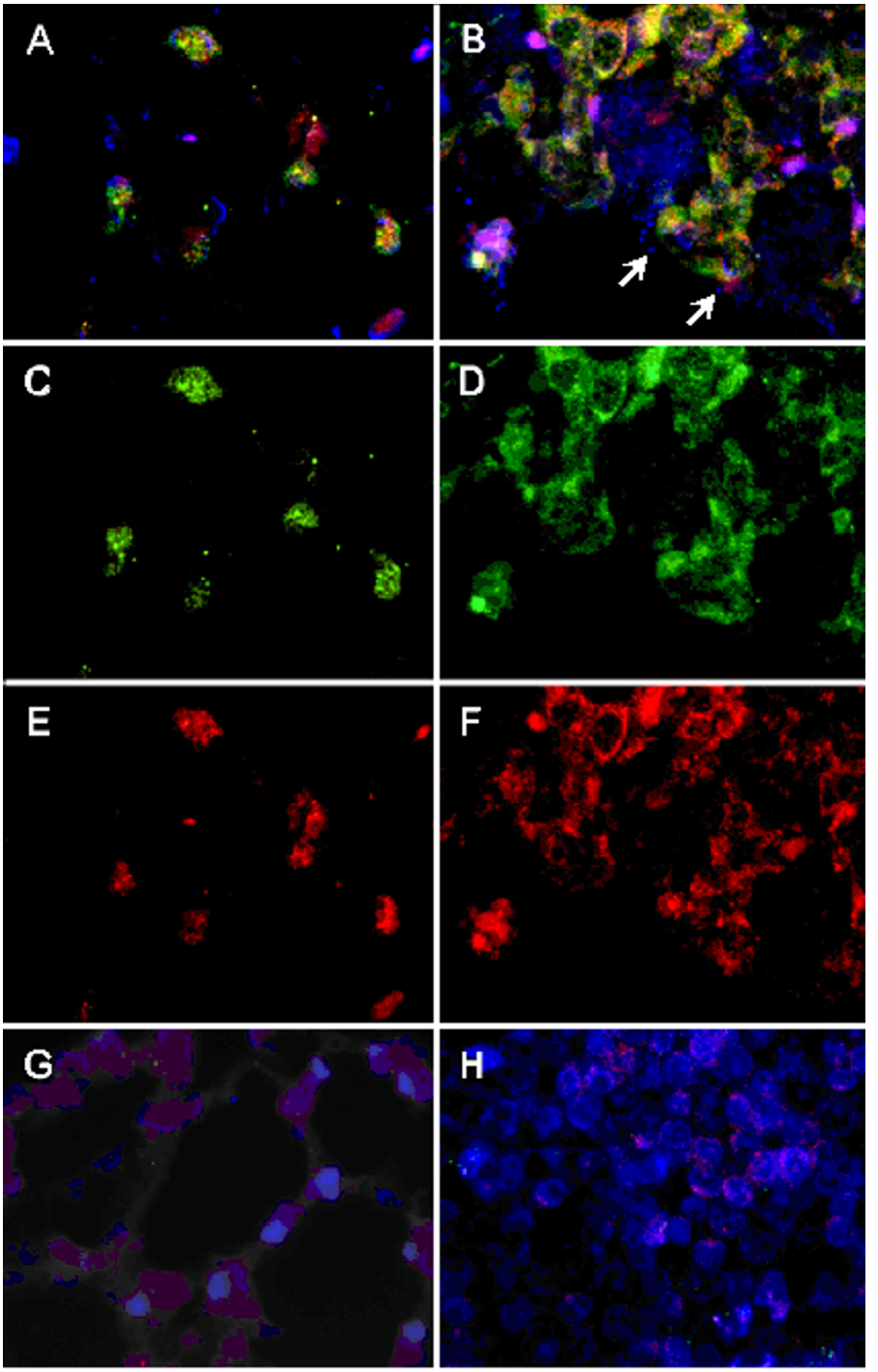

Figure 4
Double-probe Fluorescent *in situ* Hybridization Using Digoxigenin-labeled ATPase 6 and Fluorescein-labeled SP-B Riboprobes. Three micrometer-thick sections of normal rat lung (NRL) (A and G) and Pc-infected rat lung (PcIRL) (B and H) were processed for double-probe fluorescent *in situ* hybridization. Panels A (NRL) and B (PcIRL) are composite images of sections reacted with a mixture of anti-sense digoxigenin-labeled ATPase 6 and fluorescein-labeled SP-B probes. Panels C and E are split images of the composite A, and panels D and F are split images of the composite B, showing concurrence of red (ATPase 6) and green (SP-B) signal in the same cells. Panels G (NRL) and H (PcIRL) are composite images of sections reacted with both ATPase 6 and SP-B sense probes showing very little background signal. Arrows in panel B indicate the punctate DAPI-stained (blue) nuclei of *P. carinii* organisms.

unit is also an integral membrane protein associated with the F_0 sector [29]. There are approximately 10 copies of subunit 9 involved in forming the proton channel [30].

Except for ATPase 6 and A6L, which are encoded by the mitochondrial genome, all of the subunits of the F_0F_1 ATP synthase complex are encoded by nuclear genes [23]. It is not clear how expression of each of the protein subunits is regulated or how the complexes are assembled on the mitochondrial inner membrane. It has been shown that triiodothyronine (T3) increases the transcription of the ATPase 9 gene of the F_0 complex but has no effect on the transcription of the β subunit of the F_1 sector [31]. This result suggests that the two sectors of the complex are not necessarily coordinately expressed.

As mentioned above, the cells that expressed the ATPase 6 gene were found to be located at the apical areas of the alveoli and in the epithelium of the very distal parts of the respiratory tree. The apical areas are where type II pneumocytes are located, and the very distal parts of the respiratory tree are where Clara cells are located. Cells in these locations were also found to react with the SP-B probe. Since the SP-B gene is known to be expressed only in type II pneumocytes and Clara cells, the results suggest that these two types of cells are over-expressing the ATPase 6 gene in response to *P. carinii* infection.

The fact that the rat mitochondrial ATPase 6 gene was isolated from HEL cells inoculated with homogenate of *P. carinii*-infected rat lungs suggests that rat cells were present in the inoculum and survived in culture. This possibility is consistent with the nature of type II pneumocytes, as they are known to be long-lived in culture [32]. In this study, the HEL cells were incubated with the inoculum for 5 days before the cultures were processed for RNA isolation. This 5-day incubation was to allow sufficient time for *P. carinii* organisms to interact with the feeder layer (HEL cells) and for apoptosis of the short-lived rat-derived cells that were present in the inoculum. It is likely that type II pneumocytes survived for the entire 5-day period of incubation. It is also likely that some of the HEL cells died in the incubation period and that differentially expressed HEL RNAs were missed. Since no human genes were found to be over-expressed by the mRNA differential display performed in this study, the effects of *P. carinii* infection in gene expression in HEL cells or in human lungs remain to be investigated.

The significance of over-expression of the mitochondrial ATPase 6 gene in *P. carinii*-infected lung is unknown. Since type II pneumocytes transform into type I pneumocytes in response to lung injury [11], it is possible that this process requires a higher amount of ATP. To satisfy this need, these cells may increase the expression of the genes of the F_0F_1 ATP synthase complex. This hypothesis implies that the expression of all or most of the genes encoding various components of the ATP synthase complex would be up-regulated in response to *P. carinii* infection. It is also possible that ATPase 6 plays a role in other functions that also respond to *P. carinii* infection. If this were the case, genes of other components of the ATP synthase would not necessarily be up-regulated together with the ATPase 6 gene. All of these possibilities remain to be studied. It also remains to be determined whether over-expression of the ATPase 6 gene is specific for *P. carinii* infection. It is conceivable that over-expression of the ATPase 6 gene can be a diagnostic marker if it is unique to *P. carinii* infection. Studies are underway to explore these possibilities.

Conclusions

The technique of mRNA differential display was used to detect genes that have an altered expression in *Pneumocystis carinii*-infected hosts. The nucleotide sequence of one differentially displayed fragment was found to be identical to that of the gene encoding the rat mitochondrial ATPase 6, which is a subunit of the F_0F_1-ATP synthase complex. Northern blot analysis of total RNA extracted from *P. carinii*-infected rat lung versus that from mock-infected rat lung revealed that the ATPase 6 gene is over expressed during *P. carinii* infection. Cells that expressed the ATPase 6 gene were found lining the distal parts of the respiratory tree and in apical areas of alveoli by *in situ* hybridization. With a two-color fluorescent *in situ* hybridization, most cells that expressed the ATPase 6 gene were also found to express the SP-B gene, indicating that type II pneumocytes and Clara cells are the cell types responsible for the over-expression of the ATPase 6 gene in *P. carinii* infection.

Materials and Methods

Development of P. carinii infections in rats and preparation of P. carinii inoculum

To develop *P. carinii* infection, Sprague-Dawley female rats were immunosuppressed with dexamethasone and then trans-tracheally inoculated with 0.2 ml of homogenate of an infected rat lung containing 1×10^6 *P. carinii* trophozoites or with a homogenate of uninfected rat lung as controls as described previously [33]. After development of *P. carinii* infection (approximately six weeks after inoculation), rats were sacrificed, and lungs were removed and homogenized in minimal essential medium (MEM) (Life Technologies, Inc., Grand Island, New York) containing 10% fetal calf serum and 1% non-essential amino acids. Following removal of gross cellular debris by centrifugation at $400 \times g$, the number of *P. carinii*

organisms per ml of supernatant in the infected lung homogenate was determined as described previously [34].

Cell cultures

The human embryonic cell line HEL 299 (American Type Culture Collection, Rockville, MD) was used for *P. carinii* culture. When the cell monolayer was confluent, the cells were split into six 75-cm^2 flasks and grown to confluence. The medium on all 6 confluent HEL cell culture flasks was then removed. Two flasks, designated non-infected (NI) controls, were each replenished with 30 ml of fresh complete media. Two flasks, designated mock-infected (M), were inoculated with an aliquot of normal rat lung homogenate in 30 ml of complete media. The remaining two flasks, designated infected (I), were each inoculated with an aliquot of *P. carinii*-infected lung supernatant containing 7×10^5 *P. carinii* organisms per ml in 30 ml of media. These 6 flasks were then incubated in an isolation chamber containing a gas mixture of 5% O_2, 10% CO_2, and balance N_2 at 35°C for five days.

RNA extraction

RNA was isolated using the hot-acidic phenol extraction method described by Chuang *et al.* [35]. HEL cells were dislodged from the flask with a trypsin-EDTA solution, washed with DEPC-treated PBS, and then resuspended in 6 ml of ice-cold resuspension buffer [10 mM Tris-HCl (pH 7.5), 10 mM KC1, 5 mM $MgCl_2$]. Six ml of hot (70°C) lysis buffer [20 mM Tris-HCl (pH 7.4), 0.4 M NaCI, 40 mM EDTA, 1% β-mercaptoethanol, 1% SDS] and 1 ml of citrate-buffered phenol (pH 4.0) were added to each cell suspension. The resulting mixtures were boiled for 1 minute and then extracted with phenol:chloroform (1:1). The aqueous portion of the extraction was layered onto a cushion of 5.7 M CsCl in ultracentrifuge tubes. The tubes were centrifuged at 147,000 × *g* for 18.5 hours at 18°C. After ultracentrifugation, the supernatant was removed and the RNA pellets were dissolved in TE buffer [10 mM Tris-HCl (pH 8), 1 mM EDTA] and then treated with RNase-free DNase I (100 U/ml) to remove any residual DNA. To extract RNA from tissue, 100 mg of rat lung was ground in 6 ml of ice-cold resuspension buffer. The resulting homogenate was centrifuged at 400 × g for 10 minutes to remove the gross cellular debris. The supernatant was then treated as above starting with the addition of 6 ml of hot lysis buffer. Eight µg of each RNA was electrophoresed in triplicate on a formaldehyde agarose gel to check for integrity of the RNA and to perform Northern hybridizations, which were done as described previously [36].

Densitometry of Northern Blots

The intensities of hybridization signal on autoradiograms of Northern blots were determined by laser densitometry. An LKB 2202 UltroScan Laser Densitometer set in absorbance mode was used to scan 20 mm-long areas of each lane on the autoradiogram. Height, width, and area of each peak were determined by a least-squares-fit method, and the amount of absorbance over the scan length was evaluated and corrected for baseline absorbance by the LKB 2190 GelScan software package.

mRNA differential display

The RNAimage™ Kit (GenHunter Corporation, Nashville, TN) was used to detect differences between RNA samples of mock- (M) and *P. carinii*-infected (I) HEL cells. Three 20-µl reverse transcription (RT) reactions were set up per sample. Each reaction contained 200 ng of RNA, reaction buffer [25 mM Tris-HCl (pH 8.3), 37.6 mM KC1, 1.5 mM MgCh, 5 mM DTT], 20 µM of each deoxribonucleoside triphosphate (dNTP), and 0.2 µM of one of the following anchored primers: H-T_{11}-A, H-T_{11}-G, or H-T_{11}-C (H= AAGCTT). The RT reactions were performed as described in the procedure manual of the kit.

The resulting RT reaction products were used to prepare eight 20-µl PCRs per RT reaction. Each PCR included 2 µl of the RT reaction containing the primary strand cDNA, reaction buffer [10 mM Tris-HCl (pH 8.4), 50 mM KC1, 1.5 mM $MgCl_2$, 0.001% gelatin], 2 µM of each dNTP, 0.2 µM of the same anchored primer used in the RT reaction, 0.2 µM of one of the eight 13-mer arbitrary primers API-8 (Table 1), 12.5 µCi of α-[^{35}S] dATP (1270 Ci/mmole), and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA). Thermal cycling parameters included 40 cycles of denaturation at 94°C for 15 seconds, annealing at 40°C for 2 minutes, and extension at 72°C for 30 seconds. The final cycle included five additional minutes at 72°C to ensure complete extension of all products and a subsequent hold at 4°C.

Approximately one fifth of the PCR products from each reaction were electrophoresed on a 6% denaturing polyacrylamide gel to display the PCR product bands. The samples of reactions performed with the same primer pairs but different templates (M or I) were loaded into adjacent wells of the gel. An autoradiogram of the gel was obtained and analyzed to detect bands which were present in one of the paired lanes but not in the other. Each band was labeled with a three-part designation representing the anchored primer (H-T_{11}-A, H-T_{11}-G, or H-T_{11}-C), arbitrary primer (API - 8), and the number of the differential band in the lane from the well. Therefore, band A4II was found in the A-anchored primer (H-T_{11}-A) reaction with the AP4 arbitrary primer and was the second differentially displayed band in that lane. Differentially displayed bands were isolated and reamplified with the same primer pair as that used to produce the isolated differential display band. The amplified DNA was cloned into the TA cloning vector pCRII (Invitrogen,

Carlsbad, CA) and then sequenced by the Sanger method using the Sequenase (Amersham Life Science, Cleveland, OH) protocol as described previously [37].

Table 1: Sequences of the 8 Arbitrary Primers Used in the Differential mRNA Reactions.

Primer	Sequence
AP1	aagctt**GATTGCC**
AP2	aagctt**CGACTGT**
AP3	aagctt**TGGTCAG**
AP4	aagctt**CTCAACG**
AP5	aagctt**AGTAGGC**
AP6	aagctt**GCACCAT**
AP7	aagctt**AACGAGG**
AP8	aagctt**TTACCGC**

Cloning of a rat surfactant protein B gene fragment

A 202-bp portion corresponding to nucleotides 441 to 643 of the rat surfactant protein B gene (emb|X14778|RNSPB) [38] was amplified from rat lung RNA by RT-PCR and then cloned as follows. Two hundred ng of rat lung total RNA was added to a reaction which contained 10 mM Tris-HCl (pH 8.3), 50 mM KC1, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 mM of each dNTP, 5 mM dithiothreitol, 2.9 U RNasin, 20 pmoles of primer SP-BR (5'-GAATCACAGCTTGGACCCGC-3'), and 16 U MMLV reverse transcriptase. This RT reaction was incubated at 42°C for 60 minutes. The RT reaction products were mixed with 20 pmoles of primer SP-BF (5'-GACTAAGCCAGAGCAGAAGC-3') and 2 U of AmpliTaq DNA polymerase and then subjected to PCR which included 7 minutes at 94°C followed by 35 cycles of 94°C for 1 minute, 62°C for one minute, and 72°C for 1.5 minutes. The resulting RT-PCR product was cloned into TA vector pCR2.1 (Invitrogen, Carlsbad, CA). Two clones, pCR21/4SP-B and pCR22/lSP-B, which had the SP-B gene fragment in opposite orientations, were selected.

Generation and preparation of riboprobes

The ATPase 6 clones were used as templates in PCRs with primer pairs RATPase6F (5'-ACCCCCATCTCACTAATTCC-3') and RATPase6R (5'-AGTACTAGGGTAGCTCCTCC-3') to generate a 145 bp fragment to make ATPase 6 riboprobes. Similarly, a 202 bp fragment was amplified from pCR21/4SP-b with primers SP-BF and SP-BR (see above) to produce SP-B riboprobes. Each PCR product was ligated to adapters that contained an SP6 promoter sequence (SP6 adapter) in one reaction or to adapters with a T7 promoter sequence (T7 adapter) in another reaction according to the Lig'nScribe™ (Ambion, Austin, TX) protocol. The ligation products were amplified with an adapter-specific primer and either the probe-specific forward primer RATPase6F or SP-BF (for the T7 adapter ligation) or the probe-specific reverse primer RATPase6R or SP-BR (for the SP6 adapter ligation). Four PCR products were thus generated: RATPase6F-T7 adapter, SP6 adapter-RATPase6R, SP-BF-T7 adapter, and SP6 adapter-SP-BR.

The RATPase6F-T7 adapter and SP-BF-T7 adapter PCR products were used as templates for *in vitro* transcription to produce antisense riboprobes, and the SP6 adapter-RATPase6R or SP6 adapter-SP-BR PCR PCR products were used to make sense probes. The *in vitro* transcription was performed for 2 hours at 37°C in reactions containing 100 ng of template DNA, 40 U of T7 RNA polymerase (for antisense probes) or 40 U of SP6 RNA polymerase (for sense probes), 40 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 10 mM dithioerythritol, 2 mM spermidine, 10 mM NaCl, 1 mM each of ATP, GTP, and CTP, 0.65 mM UTP, 0.35 mM digoxigenin (DIG)-labeled UTP (or fluorescein-12-UTP), and 20 U RNasin (Promega, Madison, WI). After *in vitro* transcription, 20 U of RNase-free DNase I was added to digest the DNA templates, and the labeled riboprobes were precipitated with ethanol and then resuspended in 100 µl of DEPC-treated water. One µl of RNasin was added to the suspension, and the RNA concentration was determined by spectrophotometry.

in situ hybridization of rat lung tissue using riboprobes

Three-micrometer thick sections of lung tissue from dexamethasone-suppressed uninfected and *P. carinii*-infected rats were mounted on a single ProbeOnPlus slide (Fisher Scientific, Pittsburgh, PA). Processing of tissue sections for hybridization and reactions with riboprobes were performed as described previously [39]. For colorimetric detection, hybridization with the targets was revealed by reacting the tissue sections with anti-DIG-alkaline phosphatase conjugate, followed by reaction with nitroblue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and levamisole as previously described [39]. The sections were counterstained with nuclear fast red dye (0.1% nuclear fast red, 5% aluminum sulfate) and mounted in glycerin jelly (10% gelatin, 70% glycerin, 1% phenol).

Florescent *in situ* hybridizations were performed as described previously [40,41,42]. Detection of hybridized fluorescein-labeled SP-B riboprobe was accomplished by reacting the sections with rabbit anti-fluorescein antibody followed by FITC-conjugated goat anti-rabbit antibody. For detection of hybridized DIG-labeled ATPase 6 riboprobes, hybridized sections of rat lung were incubated with mouse anti-DIG antibody followed by Texas Red-

conjugated horse anti-mouse antibody and then Texas Red-conjugated rabbit anti-horse antibody.

For evaluation by fluorescence microscopy, slides were covered with antifade containing 4', 6'-diamidine-2' phenylindole dihydrochloride (DAPI), which stained the nuclei blue. Microscopic analysis of FISH images was done using an Aristoplan fluorescence microscope (Leitz, Rockleigh, NJ) with 63× and 100× oil-immersion objectives and appropriate filters. Separate gray images of the three probes (red, green, and blue) were taken using filters specific for FITC, Texas Red, and DAPI. The images were colored and superimposed using a software package by Vysis, Inc (Downers Grove, IL) to yield the final image.

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